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Control Effects of an Antibiotic Produced by *Streptomyces* sp. B25 on Tobacco Mosaic Virus and Determination of Its Molecular Structure

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ABSTRACT : The culture filtrate of *Streptomyces* sp. B25, which was identified in this experiment, was tested for the control of tobacco mosaic virus (TMV) with the susceptible tobacco cultivar, NC 82, under the field conditions following the preliminary examination of its characters for TMV control. Control efficacy of the culture filtrate against TMV infection continued over 50% up to 6 days after treatment, and its systemic effect was about 30% of the direct effect. In field conditions control efficacy of the culture filtrate against TMV infection was 95.3% at 2 weeks after TMV inoculation, and decreased to 58.3% at 3 weeks after inoculation. Five fold-dilution of the culture filtrate showed about half of the control efficacy by the stock culture filtrate. Analysis of the antibiotic material responsible for the inhibition of TMV infection through nuclear magnetic resonance experiments revealed that the antibiotic is antimycin A_1, which is firstly reported as an anti-phytoviral antibiotic in this experiment.

Key words **: *Streptomyces* sp., tobacco mosaic virus, control, antimycin A_1**

Inhibitors of plant virus infection were investigated from various higher plants, microorganisms, and some mushrooms, and a few of potent inhibitors have been isolated and characterized (Hudson, 1990; Stevens and Reynolds, 1992; Klement *et al.*, 1966; Ito *et al.*, 1992; Aoki *et al*, 1993). However, effective materials are mostly proteins or polysaccharides, which have little potential for the control of viruses in field conditions because their mass production and systemic translocation in plants are generally limited. Anti-phytoviral materials other than the macromolecules are necessary for the development of more useful and potent substances that can be used for the control of plant viruses, extending their uses for animal viruses.

With the above mentioned purpose, we have tested over 500 microbial isolates, including fungi and actinomycetes, and found an actinomycetes strain B25-producing antibiotic named as ASA (Yeo *et al.*, 1997). In this paper the identification of the actinomycetes, control effect of its culture filtrate against TMV infection, and molecular structure of the respective antibiotic were examined to confirm the identity of the microorganism and to determine the usefulness and identity of the control material.

MATERIALS AND METHODS

Identification of actinomycetes strain B25. Examination of the characters of actinomycetes strain B25 followed taxonomic studies of actinomycetes principally by the methods of Williams *et al.* (1983).

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The carbon utilization pattern of B25 was determined by the method of Shiring and Gottlieb (1966). Analysis of whole-cell hydrolyzates was performed according to the methods of Schaal (1985). Spore formation and spore surface ornamentation were observed with a scanning electron microscope and classified as described by Dietz and Mathew (1971).

Inhibitory effect on TMV infection. *Nicotiana tabacum* cv. Xanthi-nc was used for local lesion assay of TMV infection, and *N. tabacum* cv. NC 82 was used for systemic infection in field conditions. Tobacco leaves of NC 82 systemically infected with TMV (0.1 g) were ground in 20 ml of phosphate buffer (0.02 M, pH 7.3). The sap was filtered through two layers of cheesecloth. The extract was then centrifuged at 3000 rpm. for 15 min to remove debris. The supernatant solution was used as TMV inoculum.

Local lesion assay was used for examining the persistence and systemic effect of B25-producing antiviral material. For the persistence, Xanthi-nc tobacco plants at 8-10 leaf stage were treated with the ethyl acetate extract of 4-day-old B25 culture (grown at 27℃, 250 rpm) diluted in water at the concentration equivalent to the culture broth on upper half-leaf surfaces, and TMV was mechanically inoculated using 600-mesh carborundum for the periods from 0 to 8 days. The other half leaves were treated with water only and inoculated as above. At 3 days after each inoculation, local lesions formed on leaves were counted. Three replications were used for each treatment. For systemic effect, the partially purified material was applied on the lower leaf surfaces, and TMV was inoculated on the upper leaf surfaces. Local lesion assay was examined as above.

The above culture extraction solution and its 5-fold water dilution were sprayed on upper leaf surfaces of NC 82 tobacco plants to be fully wet at 2 weeks after transplanting in an experimental field located in the Korea Ginseng and Tobacco Research Institute at Taejon, and cotton swab soaked with TMV inoculum which was prepared from infected leaf sap dilution mixed with 600-mesh carborundum was rubbed on leaf surfaces (two leaves for each plant). Two and 3 weeks after inoculation, systemic mosaic symptom appearances were examined, and compared with the control with no treatment of the culture filtrate. About 30 plants were tested for each treatment.

Isolation and molecular structure of an antibiotic produced by B25. The isolation and purification of the respective antiviral material were described in the previous study (Yeo et al, 1997); however, in this study 14-day-old rice culture of B25 was used instead of broth culture because 70 g of the rice culture produced as much as 4 L broth culture. Molecular structure of the purified antibiotic was analyzed by mass spectrometry and nuclear magnetic resonance (NMR) spectrometry, and by the help of library search on antibiotics.

RESULTS

Identification of the producing strain B25. Growth characteristics of the strain B25 are presented in Table 1. Vegetative mycelia grew abundantly on yeast-malt extract, tyrosine, and nutrient agar media. The spore chains were spiral in shape and each had over 5~13 spores per chain. The spores were round in shape, 0.4 x 0.6~0.4 x 0.7 μm long in size, and had a spiny surface (Fig. 1). The

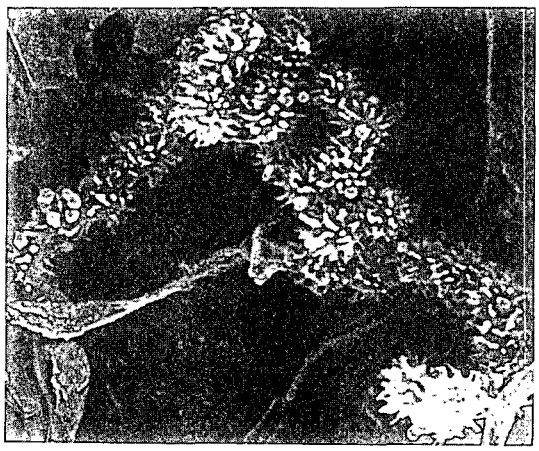

Fig. 1. Scanning electron microscopy of *Streptomyces* sp. strain B25. Note the chains of spiny spores(x 5,700).

Table 1. Cultural characteristics of *Streptomyces* sp. strain B25

Medium	Amount of growth	Color of mycelium		Soluble pigment
		Aerial	Substrate	
Yeast extract-malt extract agar (ISP No. 2)	Abundant	Gray	Dark brown	None
Oatmeal agar (ISP No. 3)	Moderate	White	Dark brown	None
Inorganic salts-starch agar (ISP No. 4)	Moderate	White/Gray	Pale yellow	None
Glycerol-asparagine agar (ISP No. 5)	Moderate	None	Brown	None
Peptone-yeast extract-iron agar (ISP No. 6)	Moderate	None	Pale yellow	None
Tyrosine agar (ISP No. 7)	Abundant	Gray	Dark brown	None
Glucose-asparagine agar	Moderate	None	Brown	None
Bennet's agar	Moderate	None	Purplish brown	None
Nutrient agar	Abundant	White	Brown	None

Table 2. Morphological and physiological characteristics of *Streptomyces* sp. strain B25

Characteristics	B25[a]
Spore morphology	Round
Spore size(㎛)	0.4×0.6-0.4×0.7
Spore surface	Spiny
Spore chain morphology	Spiral
Spore number per chain	5-13
Spore motility	None
Melanoid pigment	+
Soluble pigment	−
Diaminopimelic acid	LL
Carbohydrate utilization	
D-Glucose	+
L-Arabinose	±
D-Xylose	±
Inositol	±
D-Mannitol	±
D-Fructose	±
L-Rhamnose	−
Sucrose	−
Raffinose	−
Salicin	±
Cellobiose	±
Cellulose	−
Melibiose	±
Maltose	+

a: +: positive, −: negative, ±: intermediate

substrate mycelia were brown and aerial mass was grey-white. Melanoid pigment was produced in tyrosine-yeast extract agar. Chemical analysis of whole cell hydrolyzates of B25 demonstrated the presence of LL-diaminopimelic acid as a component of cell wall. Carbon utilization pattern and some other physiological properties are summarized in Table 2. The taxonomic characteristics described above indicates that strain B25 belongs to the genus *Streptomyces*.

Persistence and systemic activity of the B25-producing antibiotic. When the filtrate of the culture was mixed with TMV inoculum, its inhibitory efficacy against TMV infection was 95.3%; however, when TMV was inoculated after treatment of the filtrate, the inhibitory efficacy was lowered but still persisted over 50% up to 6 days after treatment (Fig. 2). Afterwards the inhibitory efficacy sharply decreased and no effect was observed at 8 days after treatment.

Systemic effect of the filtrate on inhibition of TMV infection was also noted, and the efficacy was 34% in 100 ㎍/㎖ and 20% in 10 ㎍/㎖ of the partially purified B25-producing material, which corresponded to 35% and 28% of the direct effect at the respective concentraions (Table 3).

Control of TMV in field. With treatment of the culture filtrate of B25, TMV incidence was remarkably decreased with 95.3 % of control efficacy at 2 weeks after inoculation, and that of 5-fold dilution of the culture filtrate the incidence was 56.2% (Table 4). At 3 weeks after inoculation, the control efficacy was reduced to 58.3% and 21.7% for the stock solution and 5-fold dilution, respectively.

Molecular structure of the B25-producing antiviral antibiotic. The B25-producing antiviral anti-

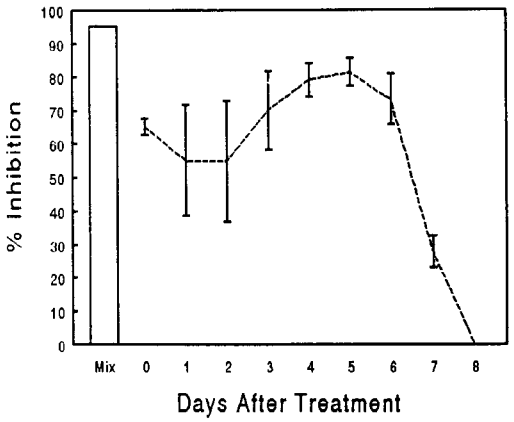

Fig. 2. Inhibitory effect of *Streptomyces* sp. strain B25 culture filtrate against TMV infection tested by inoculating TMV with days after treatment. Mix: inoculation of TMV mixed with the filtrate. Error bars are standard deviations.

Table 3. Direct and systemic Inhibitory activities of a antibiotic produced by *Streptomyces* sp. strain B25 against TMV infection on Xanthi-nc tobacco leaves

Conc. of B25-producing antibiotic(㎍/ml)	Direct inhibitory effect (A)[a] (%)	Systemic inhibitory effect (B)[b] (%)	B/A (%)
100	98	34	35
10	71	20	28

a: Upper leaf surfaces were treated with the antibiotic and inoculated with TMV
b: Lower leaf surfaces were treated with the antibiotic and respective upper leaf surfaces were inoculated with TMV.

Table 4. Control efficacy of culture filtrates of *Streptomyces* sp. strain B25 against TMV infection in field

Treatment[a]	Concentration[b]	Control efficacy after inoculation[c]	
		2 weeks	3 weeks
B25	1 x	95.3 %	58.3 %
	5 x	56.2 %	21.7 %

a: Culture filtrate of 3 days old was treated on NC 82 tobacco leaves and 1 day later infected leaf sap was inoculated on the leaves.
b: 1 x: undiluted; 5 x: 5-fold dilution
c: Control efficacy (%) = (1- TMV incidence in the treatment/TMV incidence in the control) x 100

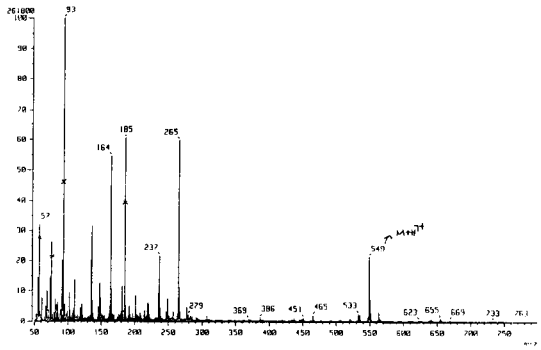

Fig. 3. Mass spectometry in FAB mode of the *Streptomyces* sp. strain B25-producing antibiotic.

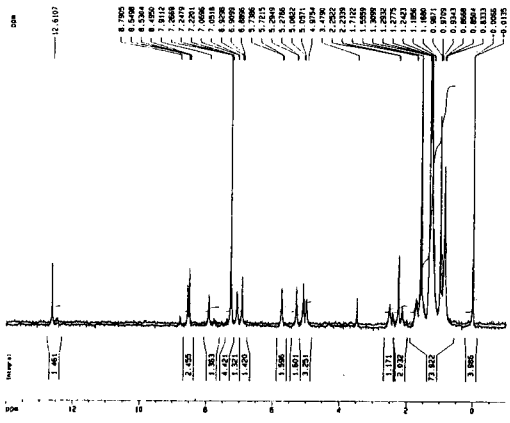

Fig. 4. ^{1}H-NMR spectrometry of the *Streptomyces* sp. strain B25-producing antibiotic.

biotic was identified as 548 dalton by the analysis of mass spectrometry in FAB mode (Fig. 3). Elucidation of the structure through ^{1}H-NMR (Fig. 4) and ^{13}C-NMR (Fig. 5) analyses with the help of library search of MW 548 antibiotic produced by *Streptomyces* sp. revealed that the respective antibiotic is antimycin A_1 (Fig. 6, Kinoshita et al., 1972).

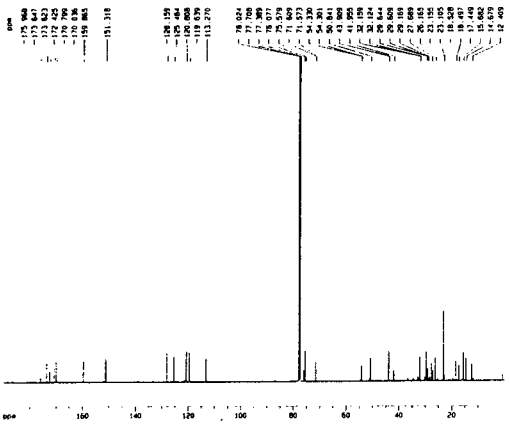

Fig. 5. ^{13}C-NMR spectrometry of the *Streptomyces* sp. strain B25-producing antibiotic.

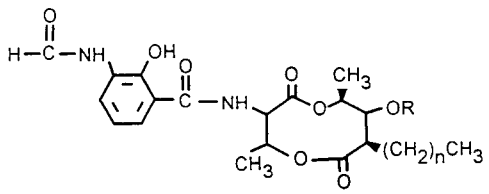

Fig. 6. Molecular structure of antimycin A_1 produced by *Streptomyces* sp. strain B25.

DISCUSSION

It has been known that higher plants and fungi widely contain antiviral substances, and some of the substances were isolated and characterized. But the antiviral substances from actinomycetes were rarely reported and inhibitory activity in the field has not been reported yet although actinomycetes is source of new metabolites (Cross, 1982).

The present study definitely shows that the *Streptomyces* sp. strain B25 (which was identified as such based on morphological and biological characteristics) contains a potent inhibitor of TMV infection on tobacco plants. The culture filtrate of this strain showed 95.5 % inhibitory activity when it was sprayed to tobacco plant 24 hours before virus inoculation in field. The field control effect of the substance produced by B25 may be related to long persistence and systemic effect of the responsible material. Although the culture filtrate showed weak systemic effects in the local lesion host under the greenhouse conditions, the activity in systemic host at the field was noteworthy. The fluctuation of inhibitory efficacies with time may result from the systemic effect in the half leaves of the control on which viral infectivity was reduced, affecting on the measurements of the relative inhibitory efficacy.

The main antiviral substance purified from rice culture of B25 was identified as antimycin A_1, which was known as one of the antifungal antibiotics with inhibitory action against certain enzyme mechanisms in the electron transport system (Schilling *et al.*, 1970). Purified antimycin A_1 applied to the upper surface of leaves of tobacco plants as a mixture of TMV also showed strong antiviral activity, and other materials isolated during its purification procedures had no antiviral effect, suggesting that the antimycin A_1 is the main compound for the antiviral effect. As for anti-phytoviral antibiotics, blasticidin S (Hirai and Shimomura, 1965) and actinomycin D (Lockhart and Semanick, 1968) were already reported. However, the antiviral effect of antimycin A_1 against TMV was firstly reported in this paper.

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